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Applicant(s) : LAMB et al.
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For : HEDGEHOG

745 Fifth Avenue
New York, NY 10151

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CLAIM OF PRIORITY

Sir:

Applicants hereby claim priority under 35 U.S.C. §119 and/or 120, from International Application No. PCT/GB02/01666 filed April 9, 2002, British Application Nos. 0108872.3 filed April 9, 2001 and 0108873.1 filed April 9, 2001. Certified copies of these applications are enclosed herewith.

Acknowledgment of the claim of priority and of the receipt of said certified copies is respectfully requested.

Respectfully submitted,
FROMMER LAWRENCE & HAUG LLP
By:
THOMAS J. KOWALSKI, Reg. No. 32,147
(212) 588-0800

INVESTOR IN PEOPLE

The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

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1. Your reference	P011299GB CLM	
2. Patent application number *(The Patent Office will fill in this part)*	**0108872.3**	09 APR 2001
3. Full name, address and postcode of the or of each applicant *(underline all surnames)*	LORANTIS LIMITED Babraham Hall, Babraham Cambridge CB2 4UL	
Patents ADP number *(if you know it)*		10APR01 E620756-10 D02246 P01/7700 0.00-0108872.3 7911 845001
If the applicant is a corporate body, give the country/state of its incorporation	GB	
4. Title of the invention	HEDGEHOG AND NOTCH	
5. Name of your agent *(if you have one)*	**D YOUNG & CO**	
"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)	**21 NEW FETTER LANE** **LONDON** **EC4A 1DA**	
Patents ADP number *(if you know it)*	**59006**	

6. If you are declaring priority from one or more earlier patent applications, give the country and date of filing of the or each of these earlier applications and *(if you know it)* the or each application number

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		Translation of Priority Documents	
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11. I/We request the grant of a Patent on the basis of this application.

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D Young & Co.

D YOUNG & CO
Agents for the Applicants

Date
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12. Name and daytime telephone number of person to contact in the United Kingdom — Catherine Mallalieu — 020 7353 4343

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Hedgehog and Notch

Field of the Invention

The present invention relates to the prevention and treatment of T cell mediated diseases or disorders.

Background

Immunological tolerance to self-antigens is vital to the proper functioning of the mammalian immune system. In addition to the deletion of self-reacting T cells in the thymus, active suppression mediated by regulatory T cells has recently been identified as an important mechanism for maintaining peripheral tolerance (WO98/20142). In autoimmune diseases such as multiple sclerosis, rheumatoid arthritis or diabetes, there is a failure of the proper regulation of tolerance. Improved treatment methods for re-establishing tolerance are desirable for autoimmune diseases. Similarly in allergic conditions and for transplantation of an organ or tissue from a donor individual, induction of tolerance to particular foreign antigens or profiles of foreign antigens is desirable.

It has recently been shown that it is possible to generate a class of regulatory T cells which are able to transmit antigen-specific tolerance to other T cells, a process termed infectious tolerance (WO98/20142). The functional activity of these cells can be mimicked by over-expression of a Notch ligand protein on their cell surfaces. In particular, regulatory T cells can be generated by over-expression of a member of the Delta or Serrate family of Notch ligand proteins. Delta or Serrate expressing T cells specific to one antigenic epitope are also able to transfer tolerance to T cells recognising other epitopes on the same or related antigens, a phenomenon termed "epitope spreading".

In addition, WO00/36089 describes a method for producing a lymphocyte or antigen presenting cell (APC) having tolerance to an allergen or antigen which method comprises incubating a lymphocyte or APC obtained from a human or animal patient with (i) a composition capable of upregulating expression of an endogenous Notch or Notch ligand in the lymphocyte and/or APC and (ii) the allergen or antigen.

Although significant advances have been made in recent years in the development of treatments for cancer, many of the resulting treatments are still based on administration of toxic compounds with consequential side-effects. As an alternative form of therapy, attempts have been made to enhance the patient's own immune response against tumours with varying degrees of success. It is not really understood how tumour cells escape recognition by the host immune system and/or inhibit an immune response. For example, it has been known for several years that a variety of immune cell-types infiltrate solid tumours in large numbers yet fail to mount an effective immunological response to the tumour. Indeed, it is possible to isolate tumour infiltrating lymphocytes which contain T cells with T cell receptors specific for tumour antigens *in vitro* which fail to be activated by tumour tissue *in vivo*. Thus there is need in the art for therapeutic methods that are capable of overcoming this immunosuppressive effect exerted by tumour cell *in vivo*.

Our co-pending PCT/GB00/04391 describes a method for enhancing the reactivity of a T cell toward a tumour cell which method comprises:

(a) isolating a T cell from a patient having said tumour cell present in their body;

(b) exposing the T cell to an agent, which agent is capable of reducing or preventing expression or interaction of an endogenous Notch or Notch ligand in the T cell; and

(c) re-introducing the T-cell into the patient;

wherein the T cell comprises a T cell receptor specific for a tumour antigen expressed by the tumour cell.

However, there remains a need in the art for the provision of further diagnostic or therapeutic compositions useful in the detection, prevention and treatment of T cell mediated diseases or disorders.

Summary of the Invention

The present invention relates to the discovery of a signalling pathway which affects the interaction between Notch proteins and their ligands and the modulation of this signalling pathway in immunotherapy. In particular, the applicant is the first to show that the Hedgehog signalling pathway affects the Notch signalling pathway. In addition, we have now shown that Shh and Ptc protein are expressed by T cells; Shh can modulate Ptc expression by T cells; Shh can modulate T cell gene expression patterns. Bhardwaj et al Nature Immun. (2001) 2:172-180 suggested that Shh is an important regulator of primitive hematopoietic cells that is dependent on downstream BMP signals, and although it is known that Hedgehog may play a role in the survival of T cells in thymus cells, we believe that we are the first to consider the peripheral immune system and to connect modulation of the Hedgehog signalling pathway with the Notch signalling pathway and the treatment of T cell mediated diseases.

Statements of the Invention

In one aspect the present invention provides a method of treatment for of T cell mediated diseases and/or diseases or disorders arising from the interaction between Notch proteins and their ligands comprising the administration of a therapeutically effective amount of a modulator of a component of a Hedgehog family member signalling pathway or a modulator of a component of a signalling pathway which is a target of Hedgehog signalling to an individual in need of the same.

In one embodiment the Hedgehog family member is Sonic hedgehog, Indian hedgehog or Desert hedgehog.

In one embodiment the pathway which is a target of Hedgehog signalling is a Wnt signalling pathway.

In one embodiment the modulator is HIP, cyclopamine, Frzb, Cerberus, WIF-1, Xnr-3, Gremlin, or Follistatin or a derivative, fragment, variant, mimetic, homologue or analogue thereof.

In another embodiment the modulator is an antibody to a component of the Hedgehog signalling pathway or an antibody to a component of the target pathway of Hedgehog signalling.

In a further embodiment the modulator is itself a component of the Hedgehog signalling pathway or a component of the target pathway of Hedgehog signalling.

The term "modulate" as used herein refers to a change or alteration in the biological activity of the Hedgehog signalling pathway or a target signalling pathway thereof. In one embodiment the modulator is an "antagonist" or "inhibitor" which blocks, at least to some extent, the normal biological activity of the Hedgehog signalling pathway. Antagonists and inhibitors may include proteins, nucleic acids and may include antibodies. In another embodiment the modulator is an agonist of the Hedgehog signalling pathway or a target signalling pathway thereof.

Various preferred features and embodiments of the present invention will now be described by way of non-limiting example and with reference to the accompanying drawings in which:
Figure 1 shows a schematic representation of HH signalling;
Figure 2 shows a schematic representation of a component of HH signalling; and
Figure 3 shows a schematic representation of Wnt signalling.
Figures 4 to 17 are discussed in more detail below.

For ease of reference a summary of the accompanying sequence listings is given below:
SEQ ID NO:1 shows the deduced amino acid sequence of mouse SHH and SEQ ID NO:2 shows the corresponding nucleic acid sequence;
SEQ ID NO:3 shows the deduced amino acid sequence of mouse Dvl-1 and SEQ ID NO:4 shows the corresponding nucleic acid sequence;
SEQ ID NO:5 shows the deduced amino acid sequence of mouse HIP and SEQ ID NO:6 shows the corresponding nucleic acid sequence; and

SEQ ID NO:7 shows the deduced amino acid sequence of mouse WIF-1 and SEQ ID NO:8 shows the corresponding nucleic acid sequence.

References and Accession Nos. are herein incorporated by reference.

Hedgehog Family Proteins

All multicellular organisms require cell communication to regulate growth and differentiation in the embryo. One strategy for this is to establish discrete organising centres that emit signals to coordinately control cell proliferation and cell fate determination. The *hedgehog (hh)* gene was identified originally through the segment polarity phenotype caused by its mutation in *Drosophila*. Genes of the *hh* family have now been isolated from several vertebrate species, including mouse, chicken, zebrafish, rat, *Xenopus* and human. The genes not only seem to show a high degree of structural homology both within and between species, but in addition exhibit some remarkable similarities in the ways in which they function in various embryonic processes. In vertebrates, *Sonic hedgehog* (*Shh*) is a key signal in several signalling centres. There are two other mammalian HH members, *Indian hedgehog (Ihh)* and *Desert hedgehog (Dhh)*.

A summary of various *hedgehog* genes is given in the following Table 1:

Table 1

Gene	**Species**
Hedgehog (hh)	*Drosophila*
Sonic hedgehog (Shh)	Mouse, Human, Rat, *Xenopus*, Chicken, Zebrafish
Indian hedgehog (Ihh)	Mouse, Human, Chicken
Desert hedgehog (Dhh)	Mouse
Banded hedgehog (X-bhh)	*Xenopus*
Cepalic hedgehog (X-chh)	*Xenopus*
Tiggy-winkle hedgehog (twhh)	Zebrafish
Echidna hedgehog (ehh)	Zebrafish

The classification of genes from different species is based on the comparison of the expression pattern and the amino acid sequence. Of all vertebrate proteins, DHH is most similar to *Drosophila* HH (51% identity over entire length of processed proteins). Amino acid identity among SHH is 93% between mouse and human, 84% between mouse and chicken, 78% between mouse and *Xenopus*, and 68% between mouse and zebrafish. Intraspecies comparison within the mouse reveals 58-63% identity in pairwise combination between SHH, IHH and DHH. Interspecies comparison between the mouse and *Xenopus* reveals highest identities between IHH and XBHH (70%) and DHH and XCHH (64%).

The various Hedgehog proteins consist of a signal peptide, with a highly conserved N-terminal region and a more divergent C-terminal domain. It is understood that the biologically active Hedgehog peptides are formed from a larger precursor protein. In addition to signal sequence cleavage in the secretory pathway, Hedgehog precursor proteins undergo an internal autoproteolytic cleavage. This autocleavage generates an N-terminal peptide (about 19kDa) and a C-terminal peptide (of about 26-28kDa). It is this N-terminal peptide that is necessary for short- and long-range Hedgehog signalling activities in *Drosophila* and vertebrates. The N-terminal peptide stays tightly associated with the surface of cells in which it is synthesised, while the C-terminal peptide is freely diffusable.

Hedgehog Signalling Pathway

Figure 1 shows one representation of a Hedgehog signalling pathway, with particular reference to signalling in vertebrates.

Epithelial cells may express the homeodomain transcription factor engrailed (*en*) and secrete Hedgehog protein shown for illustrative purposes in the Figure as Shh. We have observed that En plays an important role in the maintainance of lymphocyte survival in the peripheral immune system.

In target cells, HH signalling is mediated by two transmembrane proteins patched (Ptc) which has structural similarities to channel and transporter proteins, and Smoothened (Smo), a seven-transmembrane protein similar to G-protein coupled receptors and the Wingless receptor Frizzeled (described below). Smo is a constitutive activator of HH target genes. Its activity is normally repressed by Ptc, and this repression is relieved by HH binding to Ptc. Thus, binding of HH to Ptc allows signal transduction leading to activation of the transcription factor Gli, which is located in the nucleus of the target cells.

The signal reaches Gli through the cytoplasmic complex formed between (1) the serine/threonine kinase Fused (Fu), (2) Suppressor of Fused (SU(Fu)); and (3) Costal2 (Cos2). Signalling through this complex may be inhibited by the cAMP-dependent protein kinase A (PKA) (see Figure 2).

Gli acts on target genes wingless (Wnt) and the BMP /activin growth factors. Both Wnt and BMP are secreted to the extracellular fluid to bind to their receptors. This process is illustrated schematically in Figure 1.

A summary and comparison of components of the Hedgehog signalling pathway is given below in Table 2:

Table 2

Drosophila	Vertebrate
En	En 1,2
Hh	Ihh, Dhh, Shh
Ptc	Ptc 1,2
Smo	Smo
Ci	Gli 1-3
Target genes	
Wg	Wnt ~15
Dpp ≡ TGF_β	BMP 8-10

The nomenclature may be used interchangeably herein.

Further information on Hedgehog signalling may be find in the following articles: Ingham; Chuang and McMahon; Pepicelli *et al*; and Hammerschmidt *et al*.

Wingless/Wnt signalling pathway

We have examined the role for dysregulation of the Wnt signalling pathway in interstitial lung disease. The Wnt genes are targets of the HH pathway, and the Wnt proteins are secreted growth factors which are involved in the regulation of epithelial cell proliferation and differentiation in the lung during embryonic development.

Dishevelled-1 (Dvl-1) is the murine homolog of the fly Dsh gene and functions to transmit signals from the Wnt receptor, Frizzled, to the cytoplasm, where it regulates the kinase activity of a well known serine/threonine kinase, GSK-3b. Over expression of Dsh in fly epithelia leads to oncogenic activation of the epithelium by increasing Wnt signalling.

A representation of this pathway is shown in Figure 3. Wingless (Wg), in *Drosophila,* and, its vertebrate homolog, Wnt signalling pathways regulate cell profileration. Wg and Wnt are secreted growth factors which are involved in triggering cellular decisions. The Wg/Wnt ligand binds to Frizzled (Fz) family receptor molecules to initiate a signal transduction cascade involving the cytoplasmic protein Dishevelled (Dvl) (Sussman DJ *et al*). The GenBank accession number for *Dvl-1* cDNA is U10115. The complex illustrated in Figure 3 is present in the cytoplasm of the target cell. Generally APC blocks signalling; however, in the presence of signalling from Wnt, β-catenin is released and interacts with two transcription factors - Lef-1/TCF-1 resulting in target gene expression. Target genes of Wnt include En and therefore indirectly HH, c-myc and cyclin D1. It will be appreciated that Notch signalling is also regulated by the Wnt pathway, as DVl has been found to inhibit Notch signalling.

Notch Signalling Pathway

The Notch signalling pathway is described in WO98/20142 which is herein incorporated by reference.

Modulators

The present invention relates to the use of compounds which inhibit or block (antagonise) Hedgehog signalling. Such compounds may be seen as having the effect of downregulating the expression of Hedgehog. Similarly the present invention also relates to the use of compounds which inhibit or block (antagonise) a signalling pathway which is a target of the Hedgehog signalling pathway. Conveniently such compounds may be referred to herein as inhibitors or antagonists.

The present invention also relates to the use of compounds which increase (agonise) Hedgehog signalling. Similarly the present invention also relates to the use of compounds which increase (agonise) a signalling pathway which is a target of the Hedgehog signalling pathway. Conveniently such compounds may be referred to as upregulators or agonists.

The invention contemplates that mutations that result in loss of normal function of the regulators of the Hedgehog signalling pathway or regulators of a pathway which is a target of the Hedgehog signalling pathway in human disease states in Notch signalling is involved. Gene therapy to restore such regulatory activity would thus be indicated in treating those disease states Alternatively, it is contemplated that preventing the expression of or inhibiting the activity of such signalling pathways will be useful in treating the disease states. It is contemplated that antisense therapy or gene therapy could be applied to negatively regulate such signalling pathways.

Antagonists for each component of the signalling pathway have been identified. These may be summarised as follows in Table 3:

Table 3

Component	Antagonist
HH	Hip (Chuang and McMahon), Veratrum alkaloids and distal inhibitors of cholesterol biosynthesis (Cooper *et al*) e.g. cyclopamine (Coventry *et al*).
Wnt	Frzb (Leyns *et al*), Cerberus (Bouwmeester *et al*), Gremlin (Hsu *et al*), WIF-1 (Hsieh *et al*)
Activin	Follistatin (Iemura *et al*)

HIP (for Hedgehog-interacting protein) is a membrane glycoprotein that binds to at least all three mammalian Hedgehog proteins with an affinity comparable to that of Ptc. HIP appears to attenuate Hedgehog signalling as a result of binding to Hedgehog proteins. Such a negative regulatory feedback loop could also serve to modulate the response to any Hedgehog signal. The GenBank accession number for HIP is AF116865.

Veratrum alkaloids and distal inhibitors of cholesterol biosynthesis have been studied for more than 30 years as potent teratogens capable of inducing cyclopia and other birth defects. It has also been shown that these compounds specifically block the Shh signaling pathway (Cooper *et al*). One example of such a veraturm alkaloid is cyclopamine (11-deoxojervine), a steroid isolated from the desert plant *Veratrum californicum* (Coventry *et al*).

Frzb (Frezzled) is a secreted antagonist of Wnt signalling. Frzb contains a domain similar to the putative Wnt-binding region of the Frizzled family of transmembrane receptors, but it lacks all the transmembrane domains resulting in a putative secreted Wnt-binding protein. The GenBank accession numbers for the *Xenopus*, mouse and human Frzb cDNA sequences are U68059, U68058 and U68057, respectively.

Cerberus is a secreted protein and it has been found to be an antagonist of the Wnt signalling pathway. The GenBank accession number for the *Xenopus* Cerberus cDNA is U64831.

WIF-1 (Wnt-inhibitory factor-1) is a secreted protein which binds to Wnt proteins and inhibits their activities. GenBank accession numbers for WIF-1 are: human, AF122922; mouse, AF122923; *Xenopus*, AF122924; and zebrafish, AF122925.

Gremlin is a secreted protein and it has been found to be an antagonist of the Wnt signalling pathway. The GenBank accession numbers for Gremlin cDNA are: *Xenopus*, AF045798; chick, AF045799; human, AF045800; and mouse, AF045801.

It will also be appreciated that the antagonist or agonist may itself be a component of the Hedgehog signalling pathway, or a component of the target pathway of the Hedgehog signalling pathway. Examples of such antagonists include the negative regulators of HH signalling: Ptc, Cos2 and PKA. Examples of such agonists include the positive regulators of HH signalling Smo and Gli.

In a particularly preferred embodiment use is made of PKA. PKA has been implicated in the mechanism of Hh signal transduction because it acts to repress Hh target genes in imaginal disc cells that express Ci. Ci action as transcriptional repressor or activator is contingent upon Hedgehog-regulated, PKA-dependent proteolytic processing.

Cyclic AMP (cAMP) is a nucleotide that is generated from ATP in response to hormonal stimulation of cell-surface receptors. cAMP acts as a signaling molecule by activating A-kinase; it is hydrolyzed to AMP by phosphodiesterase (PDE). cAMP levels affect cubitus cleavage and TGF-β levels. Specifically, when cAMP levels increase, TGF-β levels decrease. In another embodiment of the invention use is made of cAMP modifiers in treatment. Such modifiers include PDE inhibitors, and beta-agonists such as the beta-adrenergic agonist. For example, it has been found that ptc 1 transcription can be induced by agents activating the cAMP signal transduction pathway. Agents which elevate intracellular cAMP levels are well known in the art and we have shown that such agents could be used in the present invention throuhg their reduction of TGF-beta production.

Immunosuppressive cytokines may also be used to modulate the Hedgehog signalling pathway. Examples include members of the TGF-β family such as TGF-β-1 and TGF-β-2, and interleukins such as IL-4, IL-10 and IL-13, IFN-γ, and FLT3 ligand.

Antisense nucleic acids (preferably 10 to 20 base pair oligonucleotides) capable of specifically binding to expression control sequences or RNA are introduced into cells (*e.g.*, by a viral vector or colloidal dispersion system such as a liposome). The antisense nucleic acid binds to the target sequence in the cell and prevents transcription or translation of the target sequence. Phosphothioate and methylphosphate antisense oligonucleotides are specifically contemplated for therapeutic use by the invention. The antisense oligonucleotides may be further modified by poly-L-lysine, transferrin polylysine, or cholesterol moieties at their 5′ end.

Also comprehended by the present invention are antibody products (*e.g.*, monoclonal and polyclonal antibodies, single chain antibodies, chimeric antibodies, CDR-grafted antibodies and antigen-binding fragments thereof) and other binding proteins (such as those identified in the assays above). Binding proteins can be developed using isolated natural or recombinant enzymes. The binding proteins are useful, in turn, for purifying recombinant and naturally occurring enzymes and identifying cells producing such enzymes. Assays for the detection and quantification of proteins in cells and in fluids may involve a single antibody substance or multiple antibody substances in a "sandwich" assay format to determine cytological analysis of HH protein levels. The binding proteins are also manifestly useful in modulating (*i.e.* blocking, inhibiting, or stimulating) interactions.

Antibodies may be generated by administering polypeptides or epitope-containing fragments to an animal, usually a rabbit, using routine protocols. Examples of such techniques include those in Kohler and Milstein.

More generally, the modulators may be selected from polypeptides and fragments thereof, linear peptides, cyclic peptides, synthetic and natural compounds including low molecular weight organic or inorganic compounds. The modulator may be derived from a biological material such as a component of extracellular matrix.

Polypeptide substances may be purified from mammalian cells, obtained by recombinant expression in suitable host cells or obtained commercially. Alternatively, nucleic acid constructs encoding the polypeptides may be introduced by transfection using standard techniques or viral infection/transduction.

Modulators for use according to the present invention may be conveniently identified using a convenient screening procedure.

One assay for identifying such modulators may involve immobilizing a component of the relevant pathway, e.g. HH, or a test protein, detectably labelling the nonimmobilized binding partner, incubating the binding partners together and determining the amount of label bound. Bound label indicates that the test protein interacts with the component.

Another type of assay for identifying modulators involves immobilizing a component of the pathway, e.g. HH, or a fragment thereof on a solid support coated (or impregnated with) a fluorescent agent, labelling a test protein with a compound capable of exciting the fluorescent agent, contacting the immobilized component with the labelled test protein, detecting light emission by the fluorescent agent, and identifying interacting proteins as test proteins which result in the emission of light by the fluorescent agent. Alternatively, the putative interacting protein may be immobilized and the component may be labelled in the assay.

Moreover, such assays for identifying modulators may involve: transforming or transfecting appropriate host cells with a DNA construct comprising a reporter gene under the control of a promoter regulated by a transcription factor having a DNA-binding domain and an activating domain; expressing in the host cells a first hybrid DNA sequence encoding a first fusion of part or all of a component of the pathway, e.g. HH or Wnt, and the DNA binding domain or the activating domain of the transcription factor; expressing in the host cells a second hybrid DNA sequence encoding part or all of a protein that interacts with said component and the DNA binding domain or activating domain of the transcription factor which is not incorporated in the first fusion; evaluating the effect of a test compound on the interaction between said component and the interacting protein by detecting binding of the interacting protein to said component in a particular host cell by measuring the production of reporter gene product in the host cell in the presence or absence of the test compound; and identifying modulating compounds as those test compounds altering production of the reported gene product in comparison to production of the reporter gene product in the absence of the modulating compound. Presently preferred for use in the assay are a *lexA* promoter to drive expression of the reporter gene, the *lacZ* reporter gene, a transcription factor comprising the *lexA* DNA binding domain and the GAL4 transactivation domain, and yeast host cells.

In a particular embodiment described in relation to Hedgehog signalling the appropriate host cell is transformed or transfected with a DNA construct comprising a reporter gene under the control of the Ptc promoter; expressing in said cells a DNA sequence encoding Hedgehog; evaluating the effect of a test compound on the interaction between HH and the Ptc promoter in a particular host cell by measuring the production of reporter gene product in the host cell in the absence and presence of the test compound; and identifying modulators as those test compounds reducing the production of the reporter gene product in comparison to production of the reporter gene product in the absence of the test compound.

Analogous assays may be used for modulators of the target pathways of Hedgehog signalling. For example, for the Wnt signalling pathway, the ability of a compound to modulate the interaction of Wnt and Fz may be determined.

Combinatorial libraries, peptide and peptide mimetics, defined chemical entities, oligonucleotides, and natural product libraries may be screened for activity as modulators in such assays.

The present invention also relates to the use of derivatives, variants, fragments, analogues, homologues and mimetics of the modulators mentioned above, including those identifiable using the assay procedures.

The term "derivative" as used herein in relation to the polypeptides of the present invention includes any substitution of, variation of, modification of, replacement of, deletion of, or addition of one (or more) amino acid residues from or to the sequence providing that the resultant protein etc., possesses the capability to agonise or antagonise the action of the signalling pathway.

The term "variant" as used herein in relation to the polypeptides of the present invention includes any substitution of, variation of, modification of, replacement of, deletion of, or addition of one (or more) amino acid residues from or to the sequence providing that the resultant protein etc., possesses the capability to agonise or antagonise the action of the signalling pathway.

The term "fragment" as used herein in relation to the polypeptides of the present invention includes a variant polypeptide which has an amino acid sequence that is entirely the same as part but not all of the amino acid sequence of the aforementioned polypeptide and possesses the capability to agonise or antagonise the action of the signalling pathway.

The term "analogue" as used herein in relation to the polypeptides of the present invention includes any peptidomimetic, i.e. a chemical compound that possess the capability to agonise or antagonise the action of the signalling pathway in a similar manner to the parent polypeptide.

The term "homologue" as used herein in relation to the polypeptides of the present invention includes a polypeptide which has the same evolutionary origin as the subject polypeptide providing that it possesses the capability to agonise or antagonise the action of the signalling pathway.

The term “mimetic” as used herein in relation to the inhibitors of the present invention includes a compound which also possesses the capability to agonise or antagonise the action of the signalling pathway in a similar manner to the parent compound.

More particularly, the term "homologue" covers identity with respect to structure and/or function providing the expression product of the resultant nucleotide sequence has the inhibitory or upregulatory activity. With respect to sequence identity (i.e. similarity), preferably there is at least 75%, more preferably at least 85%, more preferably at least 90% sequence identity. More preferably there is at least 95%, more preferably at least 98%, sequence identity. These terms also encompass allelic variations of the sequences.

Sequence identity with respect to the sequences can be determined by a simple “eyeball” comparison (i.e. a strict comparison) of any one or more of the sequences with another sequence to see if that other sequence has, for example, at least 75% sequence identity to the sequence(s).

Relative sequence identity can also be determined by commercially available computer programs that can calculate % identity between two or more sequences using any suitable algorithm for determining identity, using for example default parameters. A typical example of such a computer program is CLUSTAL. Advantageously, the BLAST algorithm is employed, with parameters set to default values. The BLAST algorithm is described in detail at http://www.ncbi.nih.gov/BLAST/blast_help.html, which is incorporated herein by reference. The search parameters are defined as follows, can be advantageously set to the defined default parameters.

Advantageously, "substantial identity" when assessed by BLAST equates to sequences which match with an EXPECT value of at least about 7, preferably at least about 9 and most preferably 10 or more. The default threshold for EXPECT in BLAST searching is usually 10.

BLAST (Basic Local Alignment Search Tool) is the heuristic search algorithm employed by the programs blastp, blastn, blastx, tblastn, and tblastx; these programs ascribe significance to their findings using the statistical methods of Karlin and Altschul (see http://www.ncbi.nih.gov/BLAST/blast_help.html) with a few enhancements. The BLAST programs were tailored for sequence similarity searching, for example to identify homologues to a query sequence. For a discussion of basic issues in similarity searching of sequence databases, see Altschul *et al* (1994) Nature Genetics 6:119-129.

The five BLAST programs available at http://www.ncbi.nlm.nih.gov perform the following tasks:

blastp - compares an amino acid query sequence against a protein sequence database.

blastn - compares a nucleotide query sequence against a nucleotide sequence database.

blastx - compares the six-frame conceptual translation products of a nucleotide query sequence (both strands) against a protein sequence database.

tblastn - compares a protein query sequence against a nucleotide sequence database dynamically translated in all six reading frames (both strands).
tblastx - compares the six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide sequence database.

BLAST uses the following search parameters:

HISTOGRAM - Display a histogram of scores for each search; default is yes. (See parameter H in the BLAST Manual).

DESCRIPTIONS - Restricts the number of short descriptions of matching sequences reported to the number specified; default limit is 100 descriptions. (See parameter V in the manual page).

EXPECT - The statistical significance threshold for reporting matches against database sequences; the default value is 10, such that 10 matches are expected to be found merely by chance, according to the stochastic model of Karlin and Altschul (1990). If the statistical significance ascribed to a match is greater than the EXPECT threshold, the match will not be reported. Lower EXPECT thresholds are more stringent, leading to fewer chance matches being reported. Fractional values are acceptable. (See parameter E in the BLAST Manual).

CUTOFF - Cutoff score for reporting high-scoring segment pairs. The default value is calculated from the EXPECT value (see above). HSPs are reported for a database sequence only if the statistical significance ascribed to them is at least as high as would be ascribed to a lone HSP having a score equal to the CUTOFF value. Higher CUTOFF values are more stringent, leading to fewer chance matches being reported. (See parameter S in the BLAST Manual). Typically, significance thresholds can be more intuitively managed using EXPECT.

ALIGNMENTS - Restricts database sequences to the number specified for which high-scoring segment pairs (HSPs) are reported; the default limit is 50. If more database sequences than this happen to satisfy the statistical significance threshold for reporting (see EXPECT and CUTOFF below), only the matches ascribed the greatest statistical significance are reported. (See parameter B in the BLAST Manual).

MATRIX - Specify an alternate scoring matrix for BLASTP, BLASTX, TBLASTN and TBLASTX. The default matrix is BLOSUM62 (Henikoff & Henikoff, 1992). The valid alternative choices include: PAM40, PAM120, PAM250 and IDENTITY. No alternate scoring matrices are available for BLASTN; specifying the MATRIX directive in BLASTN requests returns an error response.

STRAND - Restrict a TBLASTN search to just the top or bottom strand of the database sequences; or restrict a BLASTN, BLASTX or TBLASTX search to just reading frames on the top or bottom strand of the query sequence.

FILTER - Mask off segments of the query sequence that have low compositional complexity, as determined by the SEG program of Wootton & Federhen (1993) Computers and Chemistry 17:149-163, or segments consisting of short-periodicity internal repeats, as determined by the XNU program of Claverie & States (1993) Computers and Chemistry 17:191-201, or, for BLASTN, by the DUST program of Tatusov and Lipman (see http://www.ncbi.nlm.nih.gov). Filtering can eliminate statistically significant but biologically uninteresting reports from the blast output (e.g., hits against common acidic-, basic- or proline-rich regions), leaving the more biologically interesting regions of the query sequence available for specific matching against database sequences.

Low complexity sequence found by a filter program is substituted using the letter "N" in nucleotide sequence (e.g., "NNNNNNNNNNNNN") and the letter "X" in protein sequences (e.g., "XXXXXXXXX").

Filtering is only applied to the query sequence (or its translation products), not to database sequences. Default filtering is DUST for BLASTN, SEG for other programs. It is not unusual for nothing at all to be masked by SEG, XNU, or both, when applied to sequences in SWISS-PROT, so filtering should not be expected to always yield an effect. Furthermore, in some cases, sequences are masked in their entirety, indicating that the statistical significance of any matches reported against the unfiltered query sequence should be suspect.

NCBI-gi - Causes NCBI gi identifiers to be shown in the output, in addition to the accession and/or locus name.

Most preferably, sequence comparisons are conducted using the simple BLAST search algorithm provided at http://www.ncbi.nlm.nih.gov/BLAST.

Other computer program methods to determine identify and similarity between the two sequences include but are not limited to the GCG program package (Devereux *et al*

1984 Nucleic Acids Research 12: 387) and FASTA (Atschul *et al* 1990 J Molec Biol 403-410).

In some aspects of the present invention, no gap penalties are used when determining sequence identity.

The present invention also encompasses use of nucleotide sequences that are complementary to the sequences presented herein, or any fragment or derivative thereof. If the sequence is complementary to a fragment thereof then that sequence can be used as a probe to identify similar promoter sequences in other organisms.

The present invention also encompasses use of nucleotide sequences that are capable of hybridising to the sequences presented herein, or any fragment or derivative thereof.

Hybridization means a "process by which a strand of nucleic acid joins with a complementary strand through base pairing" (Coombs J (1994) Dictionary of Biotechnology, Stockton Press, New York NY) as well as the process of amplification as carried out in polymerase chain reaction technologies as described in Dieffenbach CW and GS Dveksler (1995, PCR Primer, a Laboratory Manual, Cold Spring Harbor Press, Plainview NY).

Also included within the scope of the present invention are use of nucleotide sequences that are capable of hybridizing to the nucleotide sequences presented herein under conditions of intermediate to maximal stringency. Hybridization conditions are based on the melting temperature (Tm) of the nucleic acid binding complex, as taught in Berger and Kimmel (1987, Guide to Molecular Cloning Techniques, Methods in Enzymology, Vol 152, Academic Press, San Diego CA), and confer a defined "stringency" as explained below.

Maximum stringency typically occurs at about Tm-5°C (5°C below the Tm of the probe); high stringency at about 5°C to 10°C below Tm; intermediate stringency at about 10°C to 20°C below Tm; and low stringency at about 20°C to 25°C below Tm.

As will be understood by those of skill in the art, a maximum stringency hybridization can be used to identify or detect identical nucleotide sequences while an intermediate (or low) stringency hybridization can be used to identify or detect similar or related nucleotide sequences.

In a preferred aspect, the present invention covers use of nucleotide sequences that can hybridise to the nucleotide sequences of the present invention under stringent conditions (e.g. 65°C and 0.1xSSC).

The present invention also encompasses use of nucleotide sequences that are capable of hybridising to the sequences that are complementary to the sequences presented herein, or any fragment or derivative thereof. Likewise, the present invention encompasses use of nucleotide sequences that are complementary to sequences that are capable of hybridising to the sequence of the present invention. These types of nucleotide sequences are examples of variant nucleotide sequences.

In this respect, the term "variant" encompasses sequences that are complementary to sequences that are capable of hydridising to the nucleotide sequences presented herein. Preferably, however, the term "variant" encompasses sequences that are complementary to sequences that are capable of hydridising under stringent conditions (eg. 65°C and 0.1xSSC {1xSSC = 0.15 M NaCl, 0.015 Na_3 citrate pH 7.0}) to the nucleotide sequences presented herein.

Transgenic Animals

The present invention also relates to transgenic animals which are capable of expressing or overexpressing at least one modulator useful in the present invention. Preferably the animal expresses or overexpresses HIP, Frzb-1 and/or WIF-1.

The present invention additionally relates to transgenic animals which are capable of expressing or overexpressing at least one polypeptide which is a component of the Hedgehog signalling pathway or a component of a pathway which is a target of the

Hedgehog signalling pathway, such as the Wnt signalling pathway. Preferably the animal expresses or overexpresses HH (more preferably Shh), and/or Dvl-1.

The transgenic animal is typically a vertebrate, more preferably a rodent, such as a rat or a mouse, but also includes other mammals such as human, goat, pig or cow etc.

Such transgenic animals are useful as animal models of disease and in screening assays for new useful compounds. By specifically expressing one or more polypeptides, as defined above, the effect of such polypeptides on the development of disease can be studied. Furthermore, therapies including gene therapy and various drugs can be tested on transgenic animals. Methods for the production of transgenic animals are known in the art. For example, there are several possible routes for the introduction of genes into embryos. These include (i) direct transfection or retroviral infection of embryonic stem cells followed by introduction of these cells into an embryo at the blastocyst stage of development; (ii) retroviral infection of early embryos; and (iii) direct microinjection of DNA into zygotes or early embryo cells.

The present invention also includes stable cell lines for use as disease models for testing or treatment.

A stable cell line will contain a recombinant gene or genes, also known herein as a transgene, encoding one or more inhibitors or components of a Hedgehog signalling pathway or of a pathway which is a target of the Hedgehog signalling pathway.

Preferably the transgene is HH (more preferably Shh), HIP, WIF-1, Frzb-1, Ngg and/or Dvl-1. A cell line containing a transgene, as described herein, is made by introducing the transgene into a selected cell line according to one of several procedures known in the art for introducing a foreign gene into a cell.

As also described below, the sequences encoding the modulators and components of signalling pathways, as described herein, are operably linked to control sequences, including promoters/enhancers and other expression regulation signals.

The promoter is typically selected from promoters which are functional in mammalian cells, although prokaryotic promoters and promoters functional in other eukaryotic cells may be used. The promoter is typically derived from promoter sequences of viral or eukaryotic genes. For example, it may be a promoter derived from the genome of a cell in which expression is to occur. With respect to eukaryotic promoters, they may be promoters that function in a ubiquitous manner (such as promoters of a-actin, b-actin, tubulin) or, alternatively, a tissue-specific manner (such as promoters of the genes for pyruvate kinase). Tissue-specific promoters specific for lymptocytes, dendritic cells, skin, brain cells and epithelial cells within the eye are particularly preferred, for example the CD2, CD11c, keratin 14, Wnt-1 and Rhodopsin promoters respectively. Preferably the epithelial cell promoter SPC is used. They may also be promoters that respond to specific stimuli, for example promoters that bind steroid hormone receptors. Viral promoters may also be used, for example the Moloney murine leukaemia virus long terminal repeat (MMLV LTR) promoter, the rous sarcoma virus (RSV) LTR promoter or the human cytomegalovirus (CMV) IE promoter.

It may also be advantageous for the promoters to be inducible so that the levels of expression of the heterologous gene can be regulated during the life-time of the cell. Inducible means that the levels of expression obtained using the promoter can be regulated.

In addition, any of these promoters may be modified by the addition of further regulatory sequences, for example enhancer sequences. Chimeric promoters may also be used comprising sequence elements from two or more different promoters described above.

Therapeutic Uses

A detailed description of the Notch signalling pathway and conditions affected by it may be found in our WO98/20142, WO00/36089 and PCT/GB00/04391.

Diseased or infectious states that may be described as being mediated by T cells include, but are not limited to, any one or more of asthma, allergy, graft rejection, autoimmunity, tumour induced abberrations to the T cell system and infectious diseases such as those caused by Plasmodium species, Microfilariae, Helminths, Mycobacteria, HIV, Cytomegalovirus, Pseudomonas, Toxoplasma, Echinococcus, Haemophilus influenza type B, measles, Hepatitis C or Toxicara. Thus particular conditions that may be treated or prevented which are mediated by T cells include MS, RA and diabetes. The present invention may also be used in organ transplantation or bone marrow transplantation.

We have now found that the use of modulators of Hedgehog signalling may prevent and/or promote regression of the above-mentioned diseases.

Vectors, host cells, expression

The present invention also relates to vectors which comprise a polynucleotide useful in the present invention, host cells which are genetically engineered with vectors of the invention and the production of polypeptides useful in the present invention by such techniques.

For recombinant production, host cells can be genetically engineered to incorporate expression systems or polynucleotides of the invention. Introduction of a polynucleotide into the host cell can be effected by methods described in many standard laboratory manuals, such as Davis *et al* and Sambrook *et al*, such as calcium phosphate transfection, DEAE-dextran mediated transfection, transvection, microinjection, cationic lipid-mediated transfection, electroporation, transduction, scrape loading, ballistic introduction and infection.

Representative examples of appropriate hosts include bacterial cells, such as streptococci, staphylococci, *E. Coli*, streptomyces and *Bacillus subtilis* cells; fungal cells, such as yeast cells and *Aspergillus* cells; insect cells such as *Drosophila* S2 and

Spodoptera Sf9 cells; animal cells such as CHO, COS, HeLa, C127, 3T3, BHK, 293 and Bowes melanoma cells; and plant cells.

A great variety of expression systems can be used to produce a polypeptide useful in the present invention. Such vectors include, among others, chromosomal, episomal and virus-derived vectors, e.g., vectors derived from bacterial plasmids, from bacteriophage, from transposons, from yeast episomes, from insertion elements, from yeast chromosomal elements, from viruses such as baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids. The expression system constructs may contain control regions that regulate as well as engender expression. Generally, any system or vector suitable to maintain, propagate or express polynucleotides and/or to express a polypeptide in a host may be used for expression in this regard. The appropriate DNA sequence may be inserted into the expression system by any of a variety of well-known and routine techniques, such as, for example, those set forth in Sambrook *et al.*

For secretion of the translated protein into the lumen of the endoplasmic reticulum, into the periplasmic space or into the extracellular environment, appropriate secretion signals may be incorporated into the expressed polypeptide. These signals may be endogenous to the polypeptide or they may be heterologous signals.

Polypeptides of the invention can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography is employed for purification. Well known techniques for refolding protein may be employed to regenerate active conformation when the polypeptide is denatured during isolation and/or purification.

Methods of Delivery

In the present invention the polynucleotide may be delivered to a target cell population, either *ex vivo* or *in vivo*, by any suitable Gene Delivery Vehicle.

This includes but is not restricted to, DNA, formulated in lipid or protein complexes or administered as naked DNA via injection or biolistic delivery, viruses such as retroviruses, adenoviruses, herpes viruses, vaccinia viruses, adeno associated viruses. The GDV can be designed by a person ordinarily skilled in the art of recombinant DNA technology and gene expression to express the fusion protein at appropriate levels and with the cellular specificity demanded by a particular application.

As it is well known in the art, a vector is a tool that allows or facilitates the transfer of an entity from one environment to another. In accordance with the present invention, and by way of example, some vectors used in recombinant DNA techniques allow entities, such as a segment of DNA (such as a heterologous DNA segment, such as a heterologous cDNA segment), to be transferred into a target cell. Optionally, once within the target cell, the vector may then serve to maintain the heterologous DNA within the cell or may act as a unit of DNA replication. Examples of vectors used in recombinant DNA techniques include plasmids, chromosomes, artificial chromosomes or viruses.

The vector can be delivered by viral or non-viral techniques.

Non-viral delivery systems include but are not limited to DNA transfection methods. Here, transfection includes a process using a non-viral vector to deliver a gene to a target mammalian cell.

Typical transfection methods include electroporation, DNA biolistics, lipid-mediated transfection, compacted DNA-mediated transfection, liposomes, immunoliposomes, lipofectin, cationic agent-mediated, cationic facial amphiphiles (CFAs) (Nature

Biotechnology 1996 14; 556), multivalent cations such as spermine, cationic lipids or polylysine, 1, 2,-bis (oleoyloxy)-3-(trimethylammonio) propane (DOTAP)-cholesterol complexes (Wolff and Trubetskoy 1998 Nature Biotechnology 16: 421) and combinations thereof.

Viral delivery systems include but are not limited to adenovirus vector, an adeno-associated viral (AAV) vector, a herpes viral vector, a retroviral vector, a lentiviral vector or a baculoviral vector.

Examples of retroviruses include but are not limited to: murine leukemia virus (MLV), human immunodeficiency virus (HIV), equine infectious anaemia virus (EIAV), mouse mammary tumour virus (MMTV), Rous sarcoma virus (RSV), Fujinami sarcoma virus (FuSV), Moloney murine leukemia virus (Mo-MLV), FBR murine osteosarcoma virus (FBR MSV), Moloney murine sarcoma virus (Mo-MSV), Abelson murine leukemia virus (A-MLV), Avian myelocytomatosis virus-29 (MC29), and Avian erythroblastosis virus (AEV).

A detailed list of retroviruses may be found in Coffin *et al* ("Retroviruses" 1997 Cold Spring Harbour Laboratory Press Eds: JM Coffin, SM Hughes, HE Varmus pp 758-763).

Adenoviruses and adeno-associated viruses which have good specificity for epithelial cells are particularly preferred.

Other examples of vectors include *ex vivo* delivery systems, which include but are not limited to DNA transfection methods such as electroporation, DNA biolistics, lipid-mediated transfection, compacted DNA-mediated transfection.

Thus, nucleic acid vectors according to the invention may be capable of delivery preferentially to the target cell. For example in the case of a retroviral vector, the retroviral envelope protein may be capable of directing the vector to a particular cell type or cell types. For that purpose, the envelope protein may be a modified envelope protein adapted to have a specific targeting ability, or it may be a selected envelope protein derived from a different viral or retroviral source and having the desired targeting ability.

Preferably, the nucleic acid in a vector according to the invention is operatively linked to an expression control sequence capable of causing preferential expression of the fusion protein in the target cell. The expression control sequence may be for example a promotor or enhancer which is preferentially active in certain cell types including the target cell, or a promotor or enhancer which is preferentially active under certain conditions.

The term "promoter" is used in the normal sense of the art, e.g. an RNA polymerase binding site in the Jacob-Monod theory of gene expression.

The term “enhancer” includes a DNA sequence which binds to other protein components of the transcription initiation complex and thus facilitates the initiation of transcription directed by its associated promoter.

Preferably the promoters of the present invention are tissue specific. That is, they are capable of driving transcription of a nucleic acid in one tissue while remaining largely “silent” in other tissue types. A particularly preferred promoter is the epithelial cell promoter.

The term “tissue specific” means a promoter which is not restricted in activity to a single tissue type but which nevertheless shows selectivity in that they may be active in one group of tissues and less active or silent in another group.

Administration

Compounds capable of affecting a component of the Hedgehog family signalling pathway or a target pathway thereof for use in therapy are typically formulated for administration to patients with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition. The formulation will depend upon the nature of the compound identified and the route of administration but typically they can be formulated for topical, parenteral, intramuscular, intravenous, intra-peritoneal, intranasal inhalation, lung inhalation, intradermal or intra-articular administration. The compound may be used in an injectable form. It may therefore be mixed with any vehicle which is pharmaceutically acceptable for an injectable formulation, preferably for a direct injection at the site to be treated, although it may be administered systemically.

The pharmaceutically acceptable carrier or diluent may be, for example, sterile isotonic saline solutions, or other isotonic solutions such as phosphate-buffered saline. The compounds of the present invention may be admixed with any suitable binder(s), lubricant(s), suspending agent(s), coating agent(s), solubilising agent(s). It is also preferred to formulate the compound in an orally active form.

In general, a therapeutically effective daily oral or intravenous dose of the compounds of the invention, including compounds of formula (1) and their salts, is likely to range from 0.01 to 50 mg/kg body weight of the subject to be treated, preferably 0.1 to 20 mg/kg. The compounds of the formula (I) and their salts may also be administered by intravenous infusion, at a dose which is likely to range from 0.001-10 mg/kg/hr.

Tablets or capsules of the compounds may be administered singly or two or more at a time, as appropriate. It is also possible to administer the compounds in sustained release formulations.

Typically, the physician will determine the actual dosage which will be most suitable for an individual patient and it will vary with the age, weight and response of the particular patient. The above dosages are exemplary of the average case. There can, of course, be individual instances where higher or lower dosage ranges are merited, and such are within the scope of this invention.

Alternatively, the compounds of the invention can be administered by inhalation or in the form of a suppository or pessary, or they may be applied topically in the form of a lotion, solution, cream, ointment or dusting powder. An alternative means of transdermal administration is by use of a skin patch. For example, they can be incorporated into a cream consisting of an aqueous emulsion of polyethylene glycols or liquid paraffin. They can also be incorporated, at a concentration of between 1 and 10% by weight, into an ointment consisting of a white wax or white soft paraffin base together with such stabilisers and preservatives as may be required.

For some applications, preferably the compositions are administered orally in the form of tablets containing excipients such as starch or lactose, or in capsules or ovules either alone or in admixture with excipients, or in the form of elixirs, solutions or suspensions containing flavouring or colouring agents.

The compositions (as well as the compounds alone) can also be injected parenterally, for example intracavernosally, intravenously, intramuscularly or subcutaneously. In this case, the compositions will comprise a suitable carrier or diluent.

For parenteral administration, the compositions are best used in the form of a sterile aqueous solution which may contain other substances, for example enough salts or monosaccharides to make the solution isotonic with blood.

For buccal or sublingual administration the compositions may be administered in the form of tablets or lozenges which can be formulated in a conventional manner.

For oral, parenteral, buccal and sublingual administration to subjects (such as patients), the daily dosage level of the compounds of the present invention and their pharmaceutically acceptable salts and solvates may typically be from 10 to 500 mg (in single or divided doses). Thus, and by way of example, tablets or capsules may contain from 5 to 100 mg of active compound for administration singly, or two or more at a time, as appropriate. As indicated above, the physician will determine the actual dosage which will be most suitable for an individual patient and it will vary with the age, weight and response of the particular patient. It is to be noted that whilst the above-mentioned dosages are exemplary of the average case there can, of course, be individual instances where higher or lower dosage ranges are merited and such dose ranges are within the scope of this invention.

The routes of administration and dosages described are intended only as a guide since a skilled practitioner will be able to determine readily the optimum route of administration and dosage for any particular patient depending on, for example, the age, weight and condition of the patient.

The term treatment or therapy as used herein should be taken to encompass diagnostic and prophylatic applications.

The treatment of the present invention includes both human and veterinary applications.

As used herein the terms protein and polypeptide and peptide may be assumed to be synonymous, protein merely being used in a general sense to indicate a relatively longer amino acid sequence than that present in a polypeptide, and polypetide merely being used in a general sense to indicate a relatively longer amino acid sequence than that present in a peptide. Generally for ease of reference only we will simply refer to the term polypeptide.

The invention will now be described in further detail with reference to the results shown in the figues which are summarised below:
Figure 4 shows Shh expression by human T cells;
Figure 5 shows Ptc expression in human T cells;
Figure 6 shows expression of components of the Hedgehog pathway in peripheral lymphoid tissues;
Figure 7 shows CD4 proliferation with N-Shh;
Figure 8 shows human CD8 proliferation with N-Shh;
Figure 9 shows profiles of CD69 expression with and without Shh;
Figure 10 shows profiles of CD25 expression with and without Shh;
Figure 11 shows the effect of Shh on the survival of human CD4+ T cells;

Figure 12 shows the effect of Shh on mouse CD4+ T cells;

Figure 13 shows the effect of Shh on cell cycle progression;

Figure 14 shows looks at expression of Shh, Ihh, HIP and Ptc in T cells.

Figure 15 shows induction of Shh on activated CD4+ T cells;

Figure 16 shows Shh induced T cell survival requires Gli signalling;

Figure 17 shows Shh regulation of chemokine – large spleens seen in Gli2/3-/- and single heterozygous mice as well.

We have also now shown that IL-10 and TGF-β down-regulate Shh expression.

In summary, the present inventors have surprisingly found that the following effects of Shh on T cells and the Notch signalling pathway using human and mouse studies:

- Shh downregulates Notch ligand on T cells
- Promotes survival of resting T cells
- Regulates cell-cycle entry of activated T cells
- Antibody blockage in activated T cells modulates cell-cycle entry and this shows that T cells also make Shh protein.
- Upregulates T cell activation markers such as CD69 and CD25
- Modulates expression of Shh and other signalling components as shown by PCR
- In Gli 2/3 heterozygous knockouts, Shh effect on T cells are altered and this indicates that Shh is working through a classical signalling pathway
- IL-10, TGF-β and IFN-gamma downregulation of Shh production by activated T cells
- Ab staining immunochemistry shows Shh and Ptc protein are expressed by T cells
- Shh can modulate Ptc expression by T cells
- Shh can modulate T cell gene expression patterns.

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CLAIMS

1. Use of a modulator of a Hedgehog signalling pathway, or a modulator of a pathway which is a target of the Hedgehog signalling pathway in the preparation of a medicament for treatment of a T cell mediated disease or infection.

2. Use of a modulator of a Hedgehog signalling pathway, or a modulator of a pathway which is a target of the Hedgehog signalling pathway in the preparation of a medicament for treatment of a disease or disorder associated with the Notch signalling pathway.

3. Use of claim 1 or claim 2 wherein the Hedgehog signalling pathway is the Sonic hedgehog, Indian hedgehog or Desert hedgehog signalling pathway.

4. Use of any preceding claim wherein the pathway which is a target of the Hedgehog signalling pathway is the Wnt signalling pathway.

5. Use of any preceding claim wherein the modulator is an inhibitor or upregulator of the biological activity of the Hedgehog signalling pathway.

6. Use of claim 5 in which the inhibitor is HIP, cyclopamine, Frzb, Cerberus, WIF-1, Xnr-3, Gremlin, or Follistatin or a derivative, fragment, variant, mimetic, homologue or analogue thereof.

7. Use of claim 5 in which the inhibitor is Ptc, Cos2 or PKA or an agent of the cAMP signal transduction pathway.

8. Use of any one of claims 1 to 5 in which the modulator is a member of the TGF-β family such as TGF-β-1 and TGF-β-2, an interleukin such as IL-4, IL-10 and IL-13, IFN-γ, or FLT3 ligand.

9. Use of any one of claims 1 to 5 in which the modulator is an antibody.

10. Use of any preceding claim for the preparation of a medicament for the treatment of asthma, allergy, graft rejection, autoimmunity, tumour induced abberrations to the T cell system and infectious diseases such as those caused by Plasmodium species, Microfilariae, Helminths, Mycobacteria, HIV, Cytomegalovirus, Pseudomonas, Toxoplasma, Echinococcus, Haemophilus influenza type B, measles, Hepatitis C or Toxicara.

11. Use according to claim 10 in which the autoimmune disease is multiple sclerosis, rheumatoid arthritis or diabetes.

12. A composition for use in treatment of T cell mediated diseases comprising a therapeutically effective amount of an inhibitor of a Hedgehog signalling pathway or an inhibitor of a target pathway of the Hedgehog signalling pathway and a pharmaceutically acceptable carrier, diluent or excipient.

13. A composition for use in the treatment of diseases associated with increased or decreased signalling of the Notch signalling pathway comprising a therapeutically effective amount of an inhibitor of a Hedgehog signalling pathway or an inhibitor of a target pathway of the Hedgehog signalling pathway and a pharmaceutically acceptable carrier, diluent or excipient.

ABSTRACT

HEDGEHOG AND NOTCH

Use of a modulator of a Hedgehog signalling pathway, or a modulator of a pathway which is a target of the Hedgehog signalling pathway in the preparation of a medicament for treatment of a disease or disorder associated with the Notch signalling pathway.

SEQ ID NOS: 1 & 2

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DEFINITION  M.musculus (C57BL/6J) Shh mRNA.
ACCESSION   X76290
NID         g2597987
KEYWORDS    shh gene; sonic hedgehog protein.
SOURCE      house mouse.
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SEQ 1D NOS: 3 & 4

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            homolog (Dvl-1) mRNA, complete cds.
ACCESSION   U10115
NID         g497689
KEYWORDS    .
SOURCE      mouse.
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SEQ ID NOS: 5 & 6

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DEFINITION   Mus musculus hedgehog-interacting protein (Hip) mRNA, complete cds.
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NID          g4868121
KEYWORDS     .
SOURCE       house mouse.
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             Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Mammalia;
             Eutheria; Rodentia; Sciurognathi; Muridae; Murinae; Mus.
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SEQ ID NOS: 7 & 8

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DEFINITION  Mus musculus Wnt inhibitory factor-1 mRNA, complete cds.
ACCESSION   AF122923
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KEYWORDS    .
SOURCE      house mouse.
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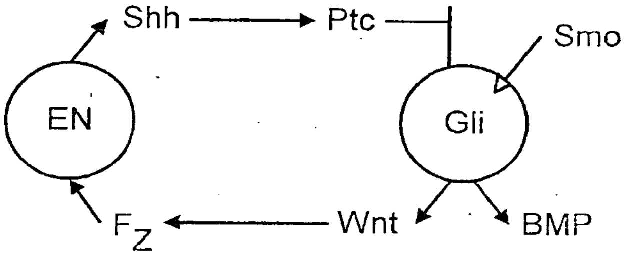
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Ptc
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BMP

FIG. 1

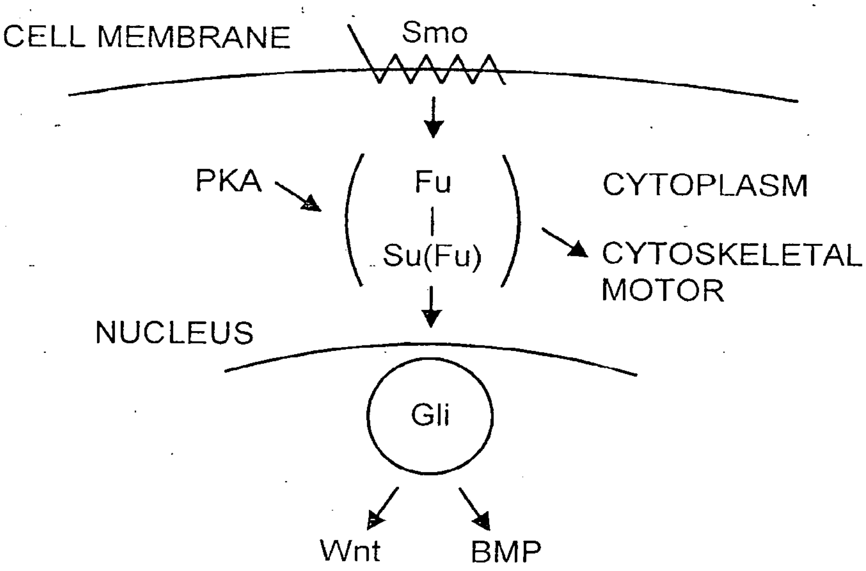

CELL MEMBRANE
Smo
PKA
Fu
Su(Fu)
CYTOPLASM
CYTOSKELETAL
MOTOR
NUCLEUS
Gli
Wnt
BMP

FIG. 2

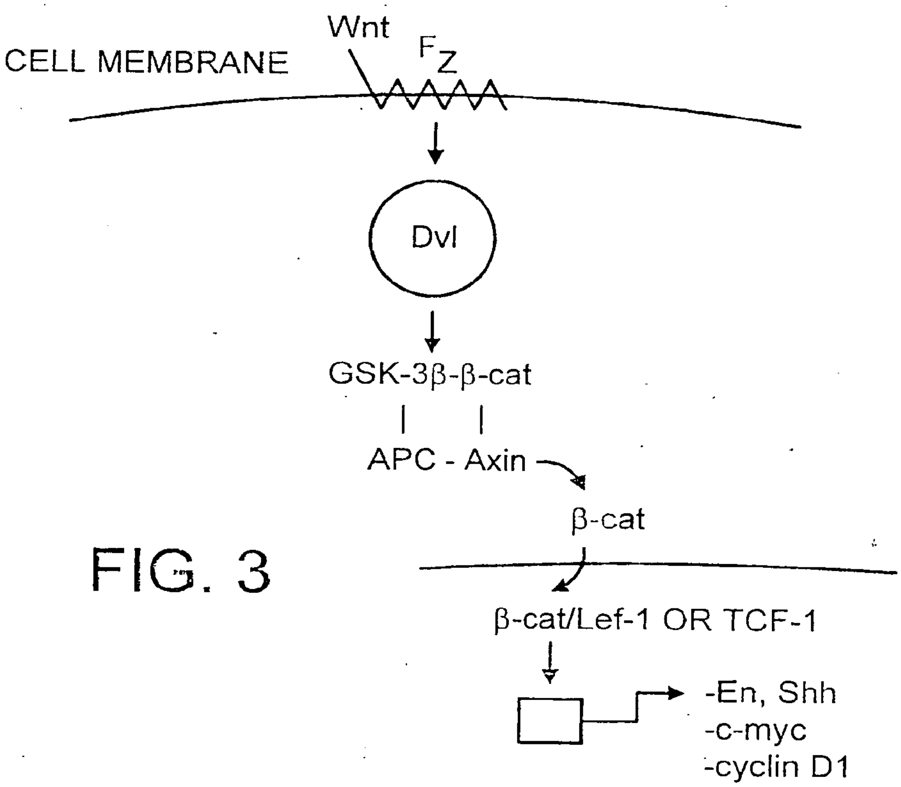
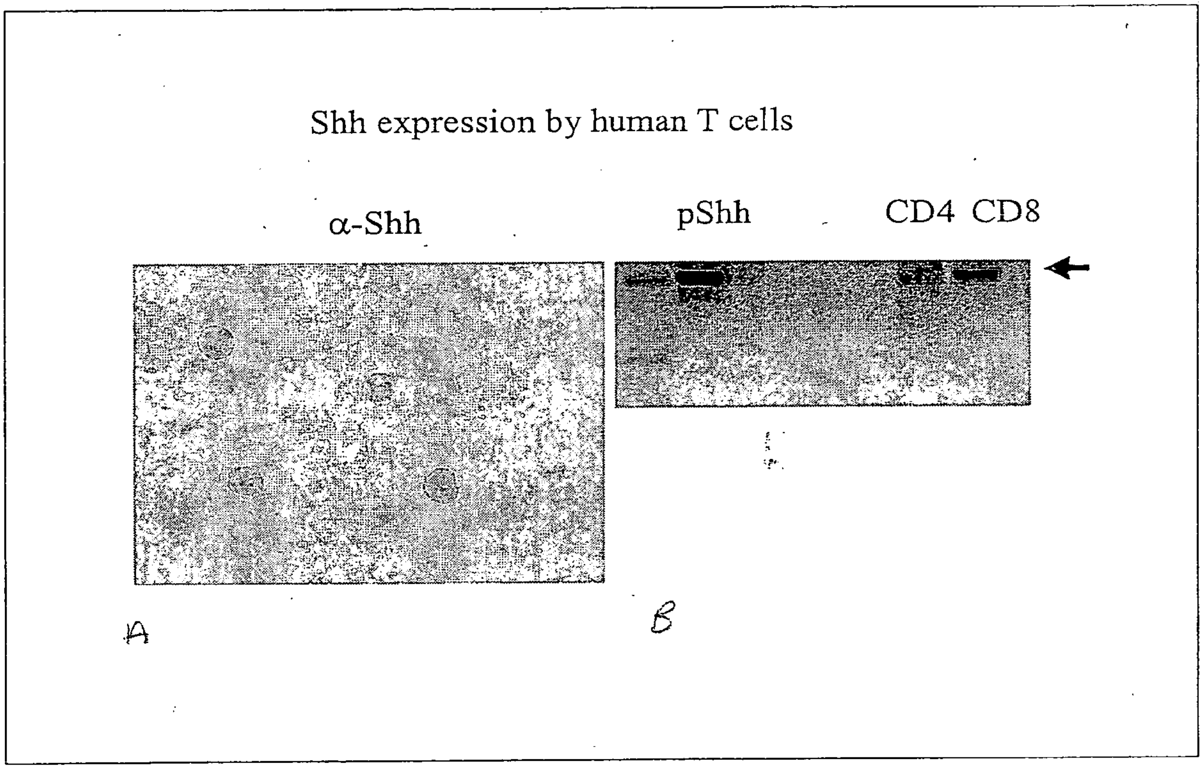

CELL MEMBRANE
Wnt
Fz
Dvl
GSK-3β-β-cat
APC - Axin
β-cat
β-cat/Lef-1 OR TCF-1
-En, Shh
-c-myc
-cyclin D1

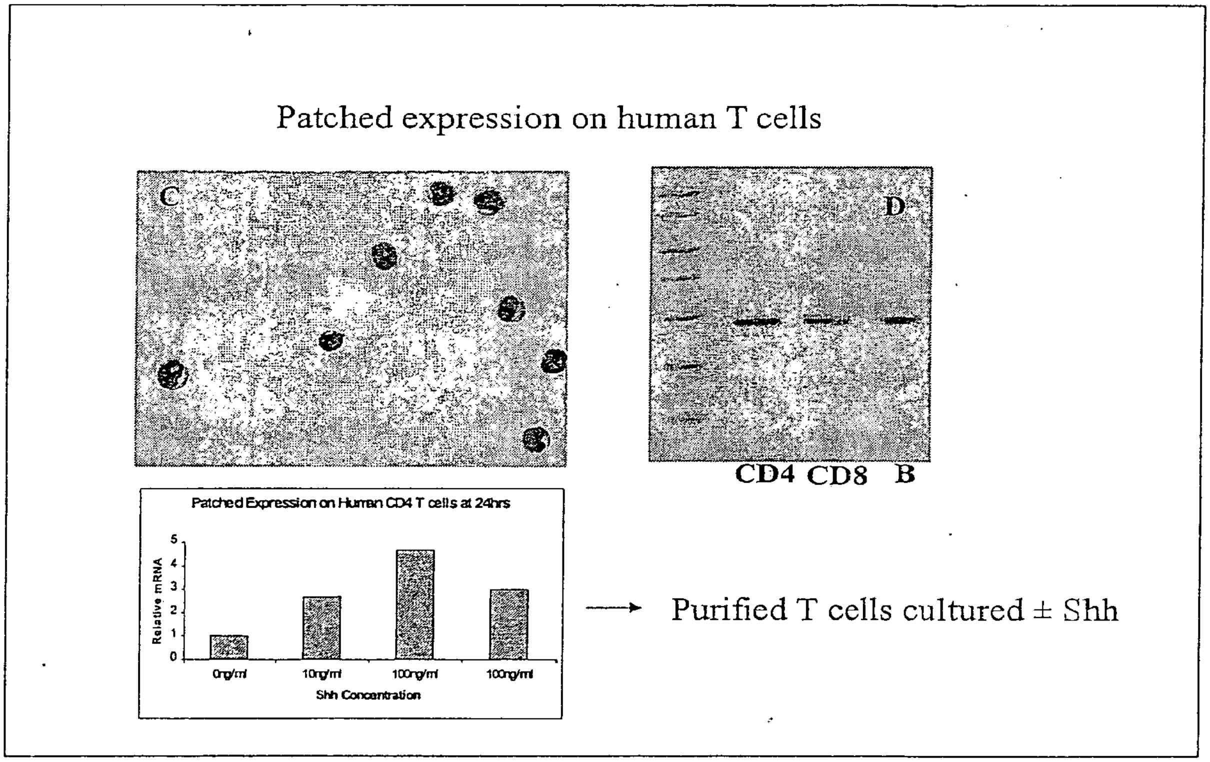
FIG. 3



A. Anti-Shh immuno-staining of unstimulated human T-cells

B. PCR expression of Shh by CD4+ and CD8+ human T-cells. pSHH = Shh plasmid control

FIGURE 4



Patched expression on human T cells

Note – right hand bar should read 1000ng/ml not 100.

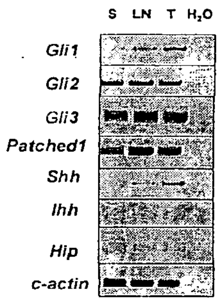
C = Antibody-staining for human Patched gene on human T-cells.

D = PCR expression (arrow) of Patched in human CD4, CD8 T-cells and B cells

Bar chart shows effect of Shh on Patched mRNA expression by cultured human CD4+ T-cells (24hr) – Taqman analyses. Unactivated human T-cells

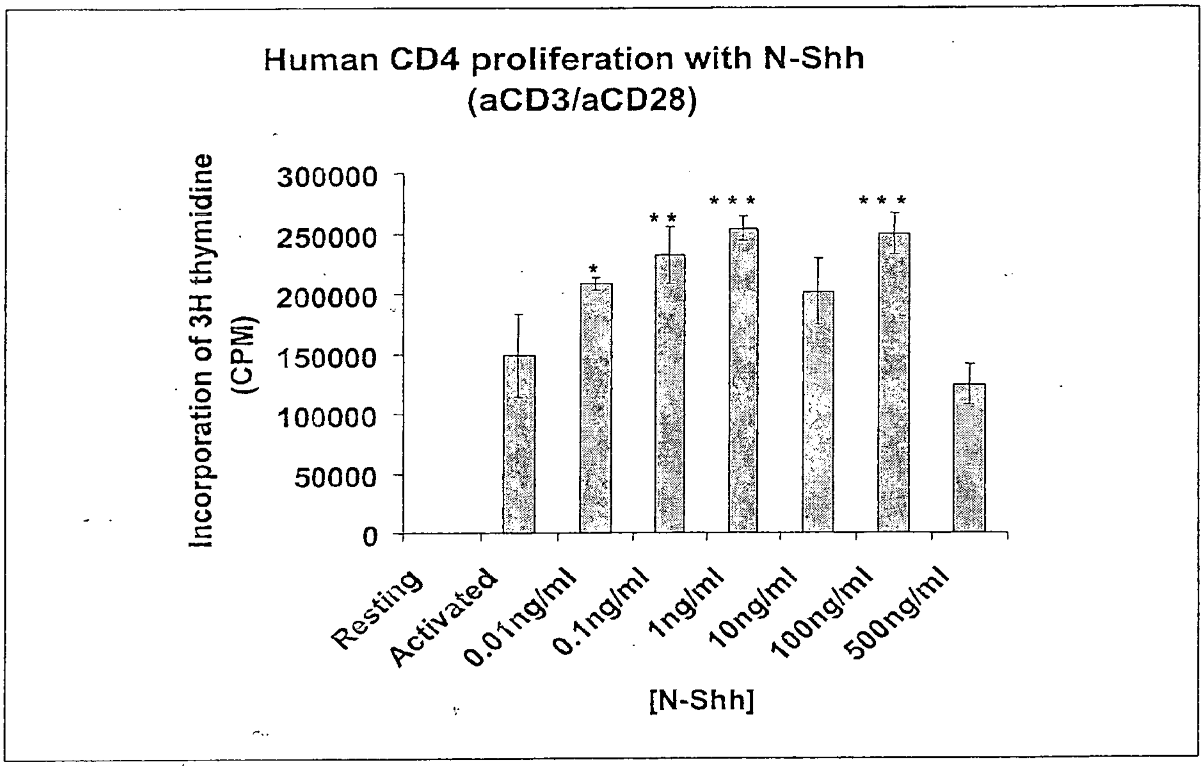
FIGURE 5

Expression of components of the Hedgehog pathway in peripheral lymphoid tissues



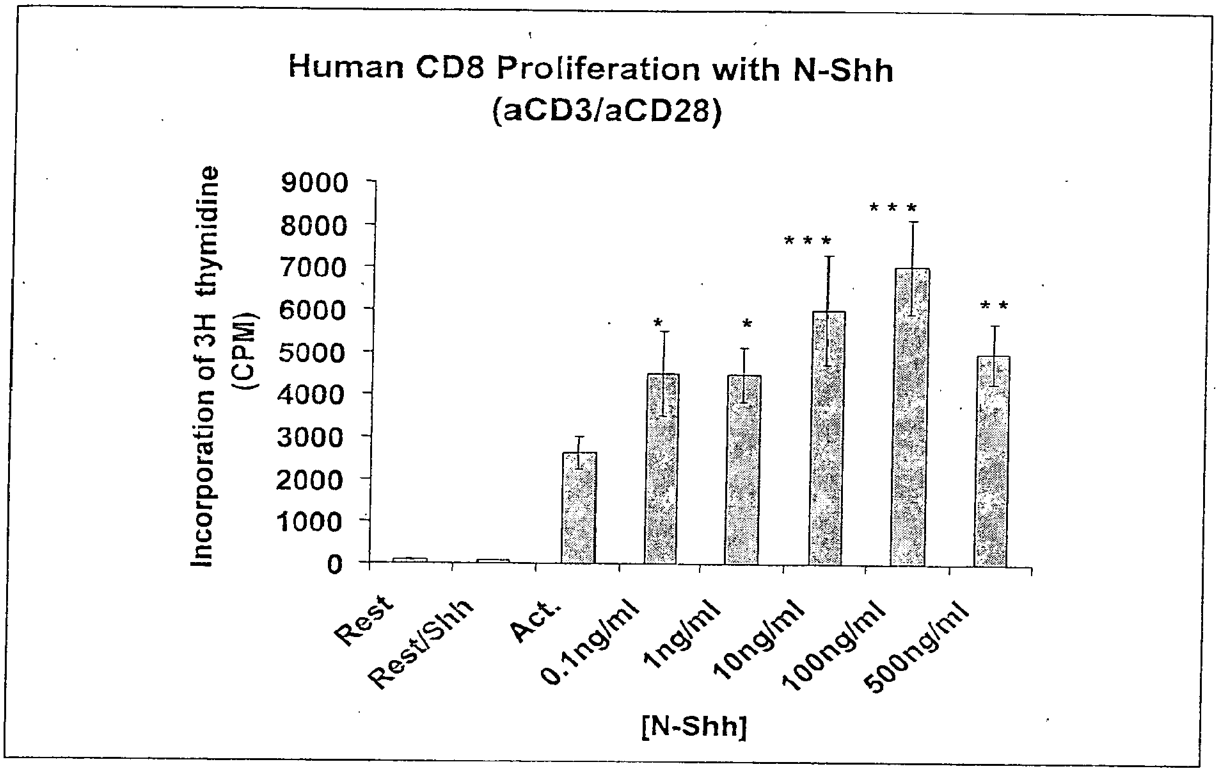
PCR analysis of hedgehog pathway genes in lymphoid tissues - spleen (S), lymph node (LN) and thymus (T). H20 = water control. SHH = Sonic hedgehog, IHH = Indian Hedgehog, HIP = hedgehog interacting protein

FIGURE 6



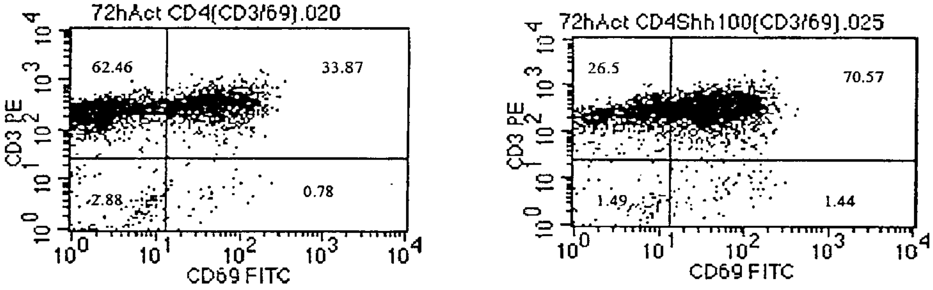
Shh (N-term active peptide – same as in all expts) significantly enhances the incorporation of 3H-thymidine in activated CD4+ T-cells – dose dependent. Could reflect either increased proliferation or increased survival or both.

FIGURE 7



Shh (N-term active peptide – same as in all expts) significantly enhances the incorporation of 3H-thymidine in activated CD8+ T-cells – dose dependent. Could reflect either increased proliferation or increased survival or both.

FIGURE 8



Activated CD4s: CD3/CD69

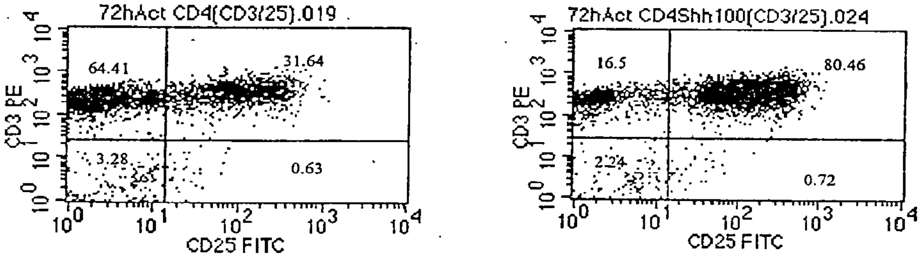
Activated only

With Shh 100ng/ml

Two-colour FACS analysis profiles of CD69 expression on CD3 positive T-cells (upper right quadrant) in CD4+ T-cells activated for 72 hour with anti-CD3 and anti-CD28 alone (activated only) or with Shh added as well at 100ng/ml. Shows Shh treatment increased CD25 expression from 33.87% to 70.57%.

FIGURE 9

Activated CD4s: CD3/CD25

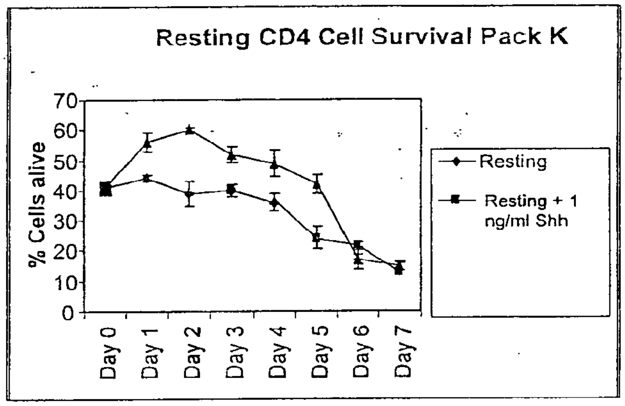


Activated only

With Shh 100ng/ml

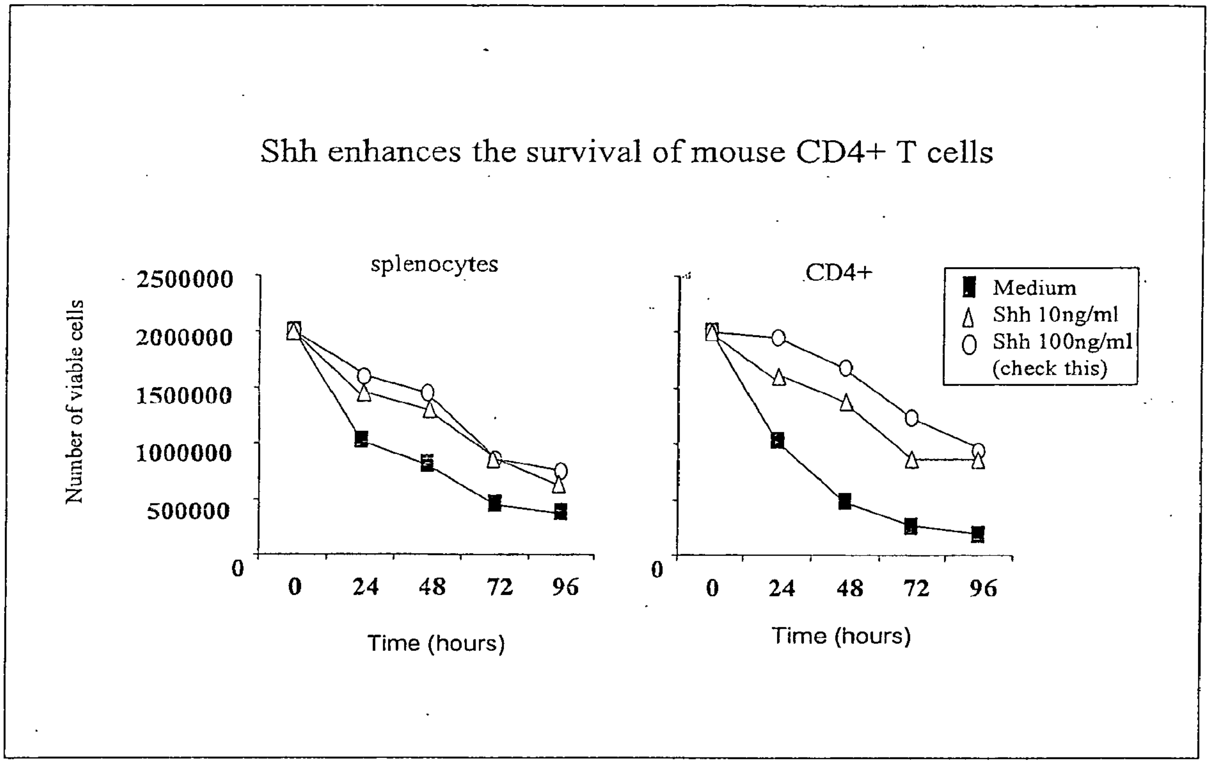
Two-colour FACS analysis profiles of CD25 expression on CD3 positive T-cells (upper right quadrant) in CD4+ T-cells activated for 72 hour with anti-CD3 and anti-CD28 alone (activated only) or with Shh added as well at 100ng/ml. Shows Shh treatment increased CD25 expression from 31.64% to 80.46%.

FIGURE 10



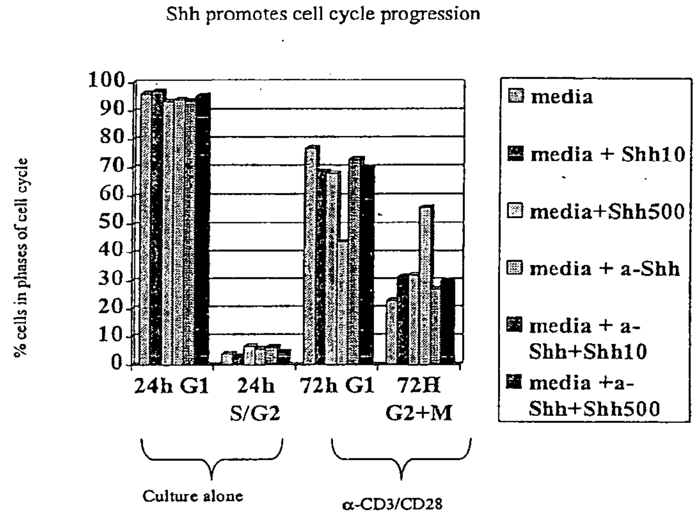
Effect of Shh on in vitro survival of human CD4+ T-cells without any other stimulus trypan blue staining of dead and alive cells in cultures.

FIGURE 11



Shh addition to mouse spleen cells and purified CD4+ T-cells in culture (no activation) enhances their survival over a 4 day time frame. NB – need to check the doses on this.

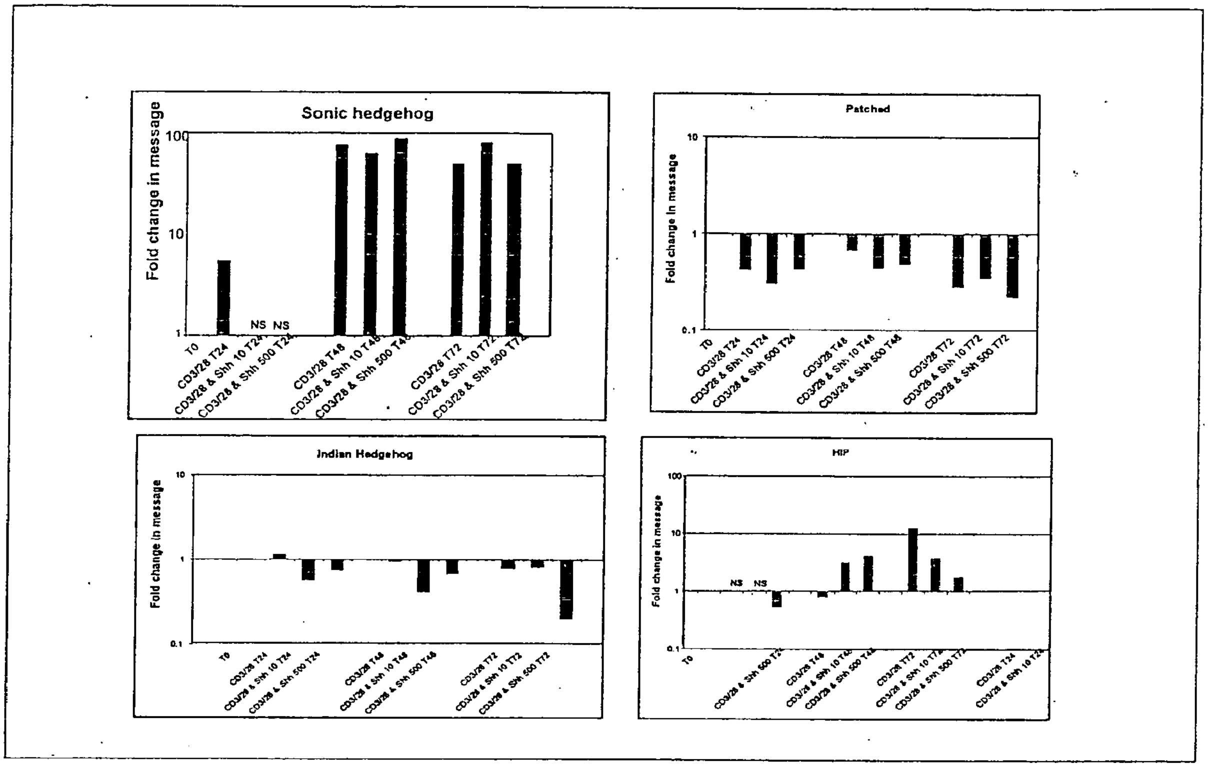
FIGURE 12



Human CD4+ T-cells cultured in medium alone or with anti-CD3/28 with or without Shh at 10 or 500ng/ml or anti-Shh neutralizing mAb, or both.

Data at 72hr shows that anti-Shh reduces % cells in G1 and increases those in G2 + M for activated cells. This indicates that Shh protein is being produced by activated T-cells (important protein follow-up to Taqman PCR data showing Shh mRNA made by activated T-cells), and that this protein is limiting the amount of cell cycle progression of the T-cell population. This effect of anti-Shh was reversed by addition of Shh to the cultures as well. Direct addition of Shh on its own had a slight effect in the opposite direction.

FIGURE 13



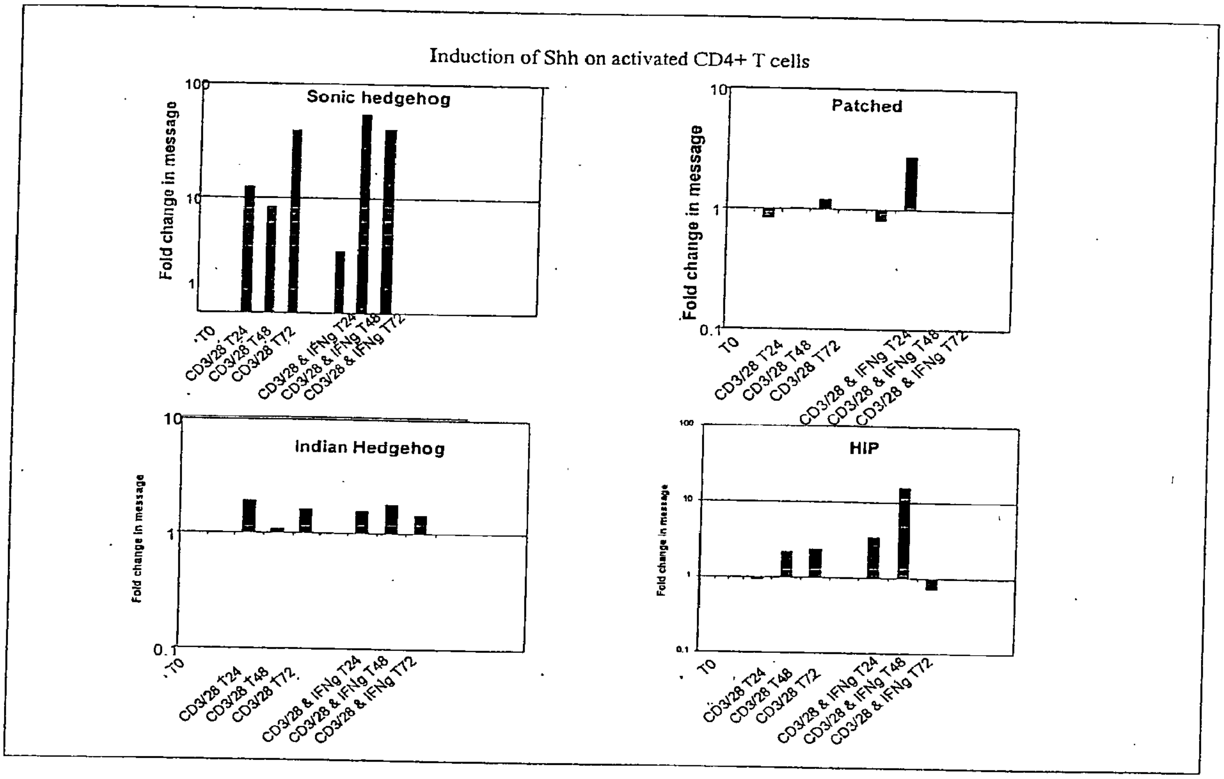
Taqman real time PCR on human CD4+ T cells +/- a-CD3/CD28 +/- Shh, looking

at expression of Shh, Ihh, Hip and Ptc

Activation of T-cells induces sustained and marked (approx 80-fold) increase in Shh mRNA peaking at 44 & 72 hr, with a down-regulation of Ptc, the receptor. At 24hr, Shh addition may down-regulate Shh mRNA expression since no signal was detected at 40 cycles of PCR (the limit of the assay).

Shh did not seem to affect the Ptc down-regulation seen. Ihh and Hip are down- and up-regulated, respectively, on addition of Shh to the activated T-cell cultures.

FIGURE 14

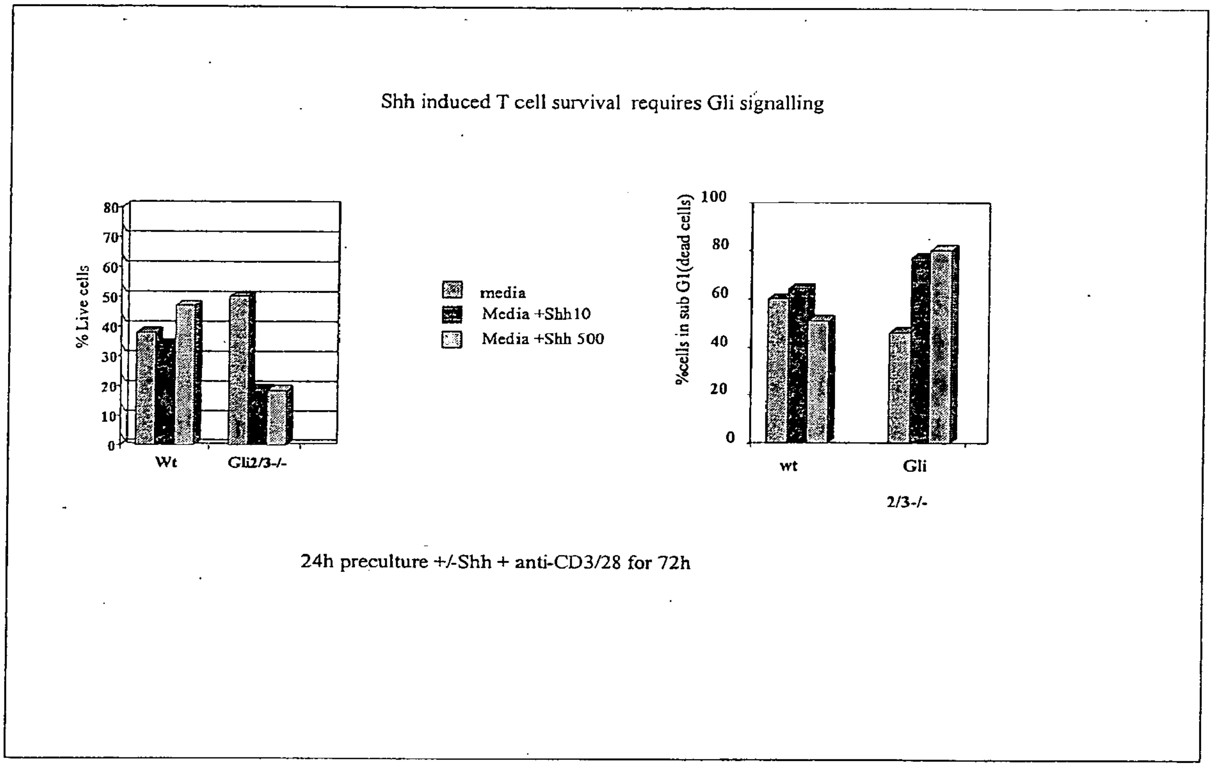


activaiton of CD4+ T cells with CD3/CD28 +/- IFN-g
lreal time PCR for Shh, Ptc, Ihh, Hip expression over time.

IFNg may modulate Shh expression by activated T-cells - down-reg early (24hr) and up-reg at 48hr. HIP was up-reg at 24 & 48hr, but appear to be down-reg by IFNg at 72hr.

Conclusion being basically, T-cell activation and response to cytokines such as IFNg can be modulated by Shh pathway.

Figure 15


Shh induced T cell survival requires Gli signalling

24h preculture +/-Shh + anti-CD3/28 for 72h

Gli2/3 -/- = mice with one allele each of Gli2 and Gli3 deleted in their genome (ie heterozygous for both these genes which are the key transcriptional mediators of hedgehog signaling).

In these mice Shh appears to promote apoptosis (less live cells and more in subG1 pool on cell cycle analysis) rather than survival. Clearly Shh can still signal but we can conclude that a partial loss of these two Gli proteins converts the signal from survival promoting to death-inducing.

This supports the conclusion that Shh is working through its appropriate signaling pathway (ie outcome is influenced by Gli levels).

Figure 16

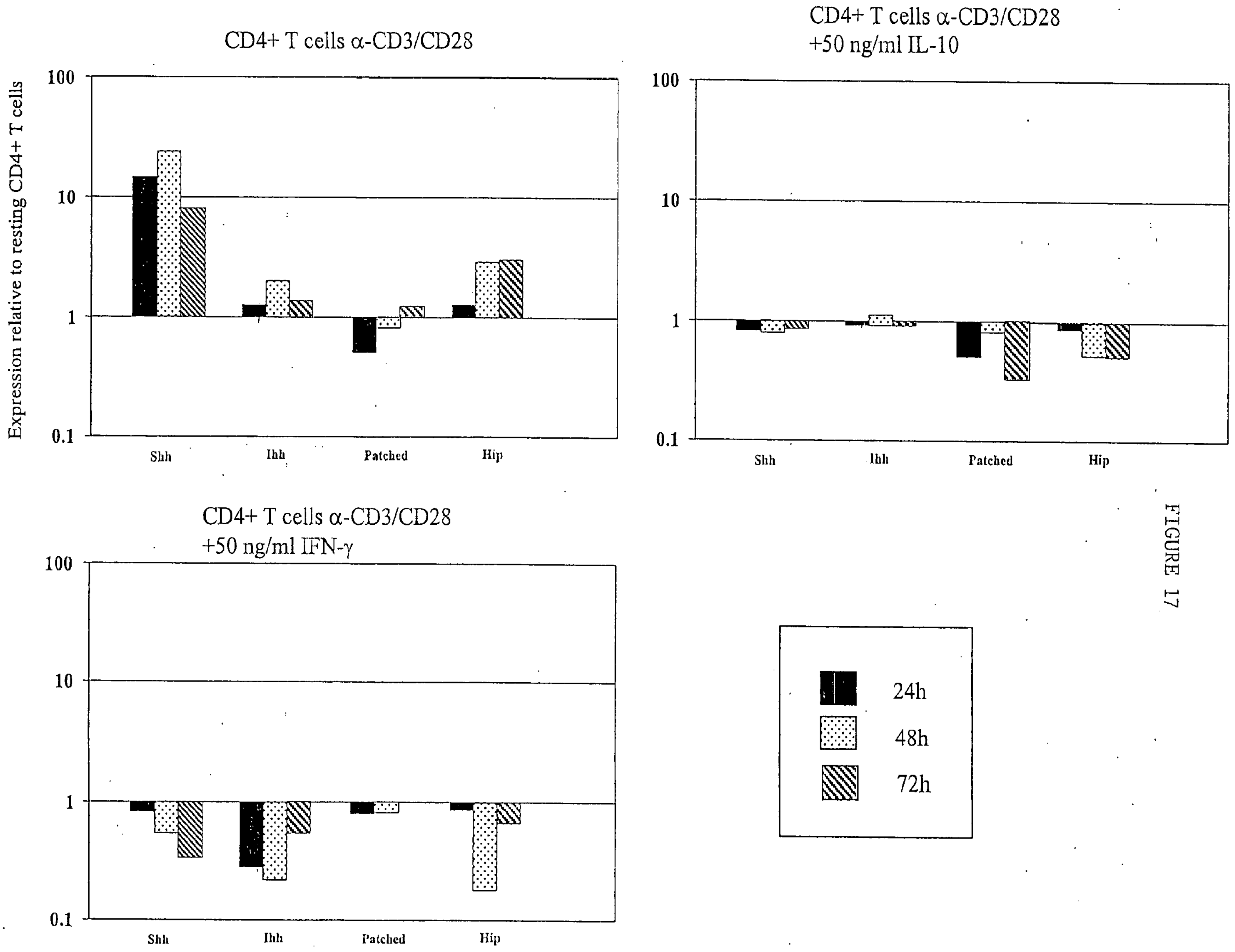

CD4+ T cells α-CD3/CD28
Expression relative to resting CD4+ T cells
100
10
1
0.1
Shh
Ihh
Patched
Hip
CD4+ T cells α-CD3/CD28
+50 ng/ml IL-10
100
10
1
0.1
Shh
Ihh
Patched
Hip
CD4+ T cells α-CD3/CD28
+50 ng/ml IFN-γ
100
10
1
0.1
Shh
Ihh
Patched
Hip
24h
48h
72h

FIGURE 17